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Solubilization of growth hormone and other recombinant proteins from *Escherichia coli* inclusion bodies by using a cationic surfactant

Nirdosh K. PURI,*† Enzo CRIVELLI,* Michael CARDAMONE,* Rodney FIDDES,‡ Joseph BERTOLINI,* Barry NINHAM§ and Malcolm R. BRANDON‡

*Bunge Scientific and Technical Services Division, 87–89 Flemington Road, North Melbourne, Victoria, Australia 3051, §Department of Applied Mathematics, Institute of Advanced Studies, Australian National University, Canberra, ACT, Australia 2601, and ‡Centre for Animal Biotechnology, School of Veterinary Science, University of Melbourne, Parkville, Victoria, Australia 3052

Recombinant pig growth hormone (rPGH) was solubilized from inclusion bodies by using the cationic surfactant cetyltrimethylammonium chloride (CTAC). The solubilizing action of CTAC appeared to be dependent on the presence of a positively charged head group, as a non-charged variant was inactive. Relatively low concentrations of CTAC were required for rapid solubilization, and protein-bound CTAC was easily removed by ion-exchange chromatography. Compared with solubilization and recovery of rPGH from inclusion bodies with 7.5 M-urea and 6 M-guanidinium chloride, the relative efficiency of solubilization was lower with CTAC. However, superior refolding efficiency resulted in final yields of purified rPGH being in the order of CTAC > urea ⩾ guanidinium chloride. Detailed comparison of the different rPGH preparations as well as pituitary-derived growth hormone by h.p.l.c., native PAGE, c.d. spectral analysis and radioreceptor-binding assay showed that the CTAC-derived rPGH was essentially indistinguishable from the urea and guanidinium chloride preparations. The CTAC-derived rPGH was of greater biopotency than pituitary-derived growth hormone. The advantages of CTAC over urea and guanidinium chloride for increasing recovery of monomeric rPGH by minimizing aggregation during refolding *in vitro* were also found with recombinant sheep interleukin-1β and a sheep insulin-like growth factor II fusion protein. In addition, the bioactivity of the CTAC-derived recombinant interleukin-1β was approximately ten-fold greater than that of an equivalent amount obtained from urea and guanidinium chloride preparations. It is concluded that CTAC represents, in general, an excellent additional approach or a superior alternative to urea and in particular guanidinium chloride for solubilization and recovery of bioactive recombinant proteins from inclusion bodies.

INTRODUCTION

The high-level expression of eukaryotic proteins in *Escherichia coli* often results in their cytoplasmic deposition as biologically inactive and insoluble aggregates known as inclusion bodies. The phenomenon of inclusion-body formation has been reported on by a number of reviewers [1–5] and it is suggested that they occur as a result of the intracellular accumulation of partially unfolded forms of the recombinant protein held as aggregates through covalent, ionic or hydrophobic interactions or combinations thereof [4,5]. However, the underlying mechanisms governing inclusion-body formation are not fully understood [3,4].

Despite the difficulties associated with solubilizing and refolding (renaturing *in vitro*) recombinant proteins deposited as inclusion bodies, there are advantages to their use as the starting material where there is a requirement for low-cost high-volume recombinant products such as growth hormones. In contrast with dealing with a soluble product, the isolation steps for inclusion bodies are relatively simple, and result in a concentrated (up to 50% of cellular protein) and relatively pure starting material that is also less susceptible to intracellular host proteinases [3,4]. However, the use of inclusion bodies as a method of production of commercially useful proteins depends on the availability of simple and economical solubilization and renaturation (downstream purification) schemes. The scientific and patent literature reports on the use of potent denaturants such as guanidinium chloride (GdmCl), NaOH, urea SDS or combinations thereof for the isolation of animal growth hormones and other recombinant proteins from inclusion bodies (reviewed in [2,6]). The major drawbacks to the large-scale use of the most popular denaturants, GdmCl and urea, have been highlighted by others [2,4,6] and include low recovery, high cost, potential irreversible modification to the protein structure (which results in a greatly decreased yield of biologically active protein) and operational difficulties associated with handling and disposal (recycling) of hazardous solubilizing agents in an ecologically permissible manner.

We report here on the results of using a readily available biocompatible cationic surfactant, cetyltrimethylammonium chloride (CTAC), as an alternative to GdmCl and urea for the solubilization and recovery of recombinant pig growth hormone (rPGH) and other recombinant proteins produced as inclusion bodies in *E. coli*.

EXPERIMENTAL

Recombinant pig growth hormone

Methionyl (1–190 amino acid) rPGH derived from plasmid pHG 935 using the gene construct and prokaryotic expression system described in U.K. patent application no. 8701848 was produced in *E. coli* as inclusion bodies and solubilized by using CTAC (ICI, Australia Pty Ltd, Melbourne, Vic., Australia).

Abbreviations used: GdmCl, guanidinium chloride; CTAC, cetyltrimethylammonium chloride; rPGH, recombinant pig growth hormone; IL-1β, interleukin-1β; IGF, insulin-like growth factor; CDA, cetyldimethylamide.

† Present address and address for correspondence and reprint requests: SmithKline Beecham Animal Health, Biological R & D – Downstream Processing Group, 601 West Cornhusker Highway, Lincoln, NE, 68521-3596, U.S.A..

Briefly, inclusion bodies were isolated by cell disruption, harvested by differential centrifugation and washed with 5 M-urea in 0.1 M-Tris/HCl buffer, pH 7.0, before use. Inclusion bodies were solubilized by using either a 5% (w/v) solution or a 1:1 ratio (w/w) of CTAC/dry weight of inclusion bodies in 0.1 M-Tris/HCl, pH 10.0, containing 1–2% (v/v) 2-mercaptoethanol for 60 min at 50 °C. The protein concentration during solubilization was 15–20 mg/ml. The solubilized inclusion bodies were clarified by centrifugation (10000 *g*; 10 min) and the supernatant was retained for analysis.

Recombinant sheep interleukin-1β (IL-1β)

Recombinant IL-1β was cloned by using the human gene as a probe and expressed in *E. coli* [7] by using the same temperature-induced bacterial expression vector as for rPGH [8]. Transformed *E. coli* from an overnight culture were grown in fresh M9 media for 4 h at 34 °C and IL-1β expression was induced by growing at 42 °C for 20 min. The cells were then grown at 34 °C for 5 h before harvesting. *E. coli* cells (equivalent to 100 ml of culture) were pelleted (10000 *g*; 10 min) and lysed in a pressure vessel at 69000 kPa. The inclusion bodies were harvested from the cell lysate by differential centrifugation (7500 *g*; 5 min) and washed in sequence with 50 mM-Tris/HCl, pH 8.5, containing 1% (v/v) Triton X-100, 0.02% (w/v) lysozyme and 2% (w/v) sodium deoxycholate for 30 min in each instance at ambient temperature. The inclusion bodies were pelleted, washed three times with 50 mM-Tris/HCl, pH 8.5, and stored at 4 °C or used immediately.

Recombinant sheep insulin-like growth factor II (IGF-II)

Recombinant IGF-II was cloned and expressed as a fusion protein with the *C*-terminus of a 26 kDa glutathione *S*-transferase enzyme using the PGEX expression system as described in [9,10]. All references to 'rIGF-II' relate to a fusion protein of IGF-II. Briefly, an overnight culture of transformed *E. coli* cells was induced with 0.1 mM-isopropyl β-D-thiogalactopyranoside and grown for 5 h at 37 °C before harvest. The presence of inclusion bodies was confirmed by using phase-contrast microscopy; the cells were lysed and the inclusion bodies recovered and treated as described above for IL-1β.

Solubilization and recovery of rPGH from inclusion bodies treated with urea, GdmCl or CTAC

Pelleted inclusion bodies [2 ml equivalent to approx. 200 mg of total protein (based on dry weight)] were solubilized in either 6 ml of 6 M-GdmCl/7.5 M-urea or 5% (w/v) CTAC [$(CH_3)_3N^+C_{16}H_{33}Cl$] in 20 mM-ethanolamine buffer, pH 10, containing 2% (v/v) 2-mercaptoethanol at a protein concentration of approx. 35 mg/ml. The inclusion-body preparations were homogenized and solubilized for 1 h at ambient temperatures or, in the case of CTAC, at 55 °C. The soluble proteins were recovered by centrifugation (2000 *g*; 5 min).

The CTAC from the solubilized rPGH preparation was removed by ion-exchange chromatography using Dowex 50WX4 (Dow Chemical Corp., Midland, MI, U.S.A.) equilibrated in 5 M-urea/0.1 M-glycine, pH 10.0. The CTAC-free rPGH (approx. 1 mg/ml) was recovered from the supernatant and dialysed into 20 mM-ethanolamine, pH 10.0, at 4 °C for 24 h.

The 6 M-GdmCl-solubilized preparation was exchanged into 7.5 M-urea, pH 10.0, and then into 3 M-urea, pH 10.0, at a protein concentration of 0.5 mg/ml using a PD-10 Sephadex G-25M column (Pharmacia Ltd.). The reduced rPGH was refolded at 4 °C for 24 h.

The 7.5 M-urea solubilized inclusion bodies were diluted to a protein concentration of 0.5 mg/ml and refolded against 3 M-urea in 20 mM-ethanolamine, pH 10.0, at 4 °C for 24 h. In all instances, refolding solutions were stirred gently at 4 °C in an open vessel to facilitate aeration and oxidation of the reduced proteins.

Purification of oxidized monomeric rPGH

The refolded protein preparations obtained using CTAC, GdmCl and urea were dialysed against 20 mM-ethanolamine, pH 10.0, at 4 °C and loaded on to a pre-equilibrated column of Whatman DE-52 ion-exchange resin (Whatman Biosystems Ltd., Maidstone, Kent, U.K.). The monomeric rPGH was differentially eluted using 30 mM-NaCl in 20 mM-ethanolamine, pH 10.0, at 4 °C.

Solubilization of rIL-1β and rIGF-II from inclusion bodies with CTAC, urea and GdmCl

Briefly, 100 μl of pelleted inclusion bodies were solubilized in either 1 ml of 6 M-GdmCl or 7 M-urea or 5% (v/v) CTAC in 50 mM-Tris/HCl buffer containing 2% (v/v) 2-mercaptoethanol for 1 h at ambient temperature or, in the case of CTAC, at 50 °C. The soluble proteins were recovered by centrifugation (10000 *g*; 15 min) and after removal of CTAC the total protein concentrations of each preparation were determined by the Bio-Rad (Bradford) Coomassie Blue protein assay. A 20 μl sample of each solubilized preparation was also precipitated with 1 ml of ethanol at −20 °C and analysed by SDS/PAGE under reducing conditions.

The 7 M-urea-solubilized preparations were renatured (refolded) at one or two different protein concentrations by dialysing against 3 M-urea in 50 mM-Tris/HCl buffer, pH 10.0, overnight at 4 °C, followed by another overnight dialysis against Tris buffer alone.

The 6 M-GdmCl-solubilized preparations were refolded by dialysis against either 2 M-GdmCl or 3 M-urea and finally against aqueous buffer as described above.

The CTAC-solubilized preparations were, after removal of CTAC, renatured in a single step by dialysing against 50 mM-Tris/HCl buffer (as above), without adjusting protein concentration.

Removal of bound CTAC from recombinant proteins

Where required, CTAC, free in solution or bound to protein, was removed by ion-exchange chromatography. Solubilized protein in CTAC was either directly mixed or diluted with 8 M-urea and mixed in batch mode with Dowex 50WX4 cation-exchange resin (Dow Chemical Corporation) equilibrated in 0.1 M-glycine/HCl and 5 M-urea, pH 10.0. The surfactant-free protein was recovered from the supernatant.

Solubilization of inclusion bodies with cetyldimethylamine (CDA) and CTAC

Inclusion bodies were solubilized using a 30% (w/v) stock solution of CTAC or a 99.6% (w/v) stock solution of CDA in 0.1 M-Tris/HCl, pH 10.0, containing 2% (v/v) 2-mercaptoethanol at 50 °C for 1 h. The final concentration of surfactant used varied from 0 to 10% (w/v). Inclusion bodies were solubilized to give protein concentrations of 40 mg/ml. The solubilized material was clarified by centrifugation and the protein (rPGH) concentration in the supernatant determined by h.p.l.c. Briefly, 50 μl samples of the supernatant were analysed by h.p.l.c. and protein concentration was estimated by using a standard curve of peak area against concentration of rPGH in mg/ml. Samples were analysed in duplicate.

H.p.l.c. analysis

Reversed-phase h.p.l.c. analysis was performed using C_1 alkyl-bonded silica columns (TSK-TMS 250; Toyo Soda Manufacturing Co., Tokyo, Japan). Elution was performed with

water/acetonitrile mixtures containing 0.1% trifluoroacetic acid with a stepwise gradient of buffer A (0.1% trifluoroacetic acid) to buffer B (100% acetonitrile in 0.1% trifluoroacetic acid). Detection was at 214 nm, with a flow rate of 1.5 ml/min.

C.d. analysis of rPGH preparations

C.d. measurements were made at room temperature using an AVIV 60DS c.d. spectrophotometer. Protein concentrations of 0.1–0.2 mg/ml in 50 mM-phosphate buffer, pH 7.5, were used for rPGH preparations. Protein concentrations were standardized on the basis of absorbance at 280 nm by using an absorption coefficient of 20500 $M^{-1} \cdot cm^{-1}$ for rPGH. A quartz cell of path length 1 mm was used for measurements. The mean residue ellipticities (θ) in degrees $\cdot cm^2 \cdot dmol^{-1}$ were calculated using a mean residue mass of 114.1 for rPGH and 115.2 for pituitary-derived growth hormone. The secondary-structure content was calculated as described in ref. [11].

SDS/ and non-SDS/PAGE and densitometer scanning of gels

SDS/PAGE was performed using 12.5% or 15% gels under both reducing and non-reducing conditions using the discontinuous system. Native (non-SDS) gels were run as for SDS-containing gels except that no SDS was used and samples were not boiled before electrophoresis. Laser-densitometer analysis of Coomasie-stained gels was performed by using a Zenith Soft Laser scanning densitometer (model SLR-TRFF) and a one-dimensional scanning software program from Biomed Instruments, Fullerton, CA, U.S.A.

Radioreceptor assay of rPGH preparations

Iodination-grade purified pituitary-pig growth hormone (UCB Bioproducts, Brussels, Belgium; batch 004, 3.5 i.u./mg) was labelled using Iodogen (Pierce Chemicals). Briefly, 10 μg of hormone was incubated with 1 μg of Iodogen in 40 μl of 50 mM-sodium phosphate buffer, pH 7.5, together with 1 mCi of ^{125}I (IMS-30; 100 mCi/ml, Amersham, Sydney, Australia) for 10 min at room temperature. Radiolabelled hormone was purified by Sephadex G-100 column chromatography (Pharmacia, Sydney, Australia) in 0.2% BSA/50 mM-sodium phosphate buffer. The specific activity of the labelled growth hormone was 50 μCi/μg.

Plasma-membrane fractions were prepared from the liver of a young adult pig collected within 5 min of slaughter and kept frozen at −20 °C. Microsomal fractions were prepared by differential centrifugation as described previously [12]. Just before use, they were stripped of endogenously bound hormone by brief treatment with $MgCl_2$ [13]. The activity of various rPGH preparations was measured in a competitive binding assay with ^{125}I-labelled pituitary hormone. Microsomal preparation (100 μl equivalent to 100 mg of the original liver wet weight) was incubated with the ^{125}I-labelled growth hormone (100000 c.p.m.) in 200 μl of assay buffer (25 mM-Tris/HCl, pH 7.4, containing 10 mM-$MgCl_2$/0.2% BSA and 0.02% benzamidine) together with various amounts of the homologous or the CTAC-, urea- or GdmCl-derived DE-52-purified monomeric rPGH preparations.

Bioactivity testing of rIL-1β preparations

The NOB-1 CTL cell assay [14] was used for assessment of the bioactivity of rIL-1β preparations. Briefly, NOB-1 cells were washed three times in RF10 media, resuspended at 2×10^6 cells/ml and 0.1 ml was added to U-bottomed microtitre plates with 0.1 ml of appropriately diluted rIL-1β preparations. After incubation for 24 h at 37 °C, plates were centrifuged at 1000 *g* and 50 μl of supernatant was transferred to a replicate flat-bottomed microtitre plate together with 50 μl (5×10^3) of CTL cells. The proliferation of CTL cells was measured by pulsing cells with [^{3}H]thymidine after 20 h incubation. Cells were harvested 4 h after pulsing and processed for radioactivity counting using an automated cell harvester. All test samples were assayed in duplicate. The relative potency (end-point titre) of rIL-1β preparations was based on the dilution of rIL-1β that gave 50% of the maximal counts incorporated.

Hypophysectomized rat tibial assay

The biological activity of growth hormone preparations was assessed using the hypophysectomized rat tibial assay [15]. Briefly, 5–100 μg of rPGH or pituitary-derived pig growth hormone per day in 0.9% NaCl was injected subcutaneously at 24 h intervals into eight hypophysectomized rats per dose group for 4 days. At 24 h after the last injection the rats were killed and the right tibia isolated. The bone was cleaned and split at the proximal end in the mid-sagittal plane. The epiphyseal cartilage was distinguished from surrounding bone after staining with silver nitrate. The sections were photographed and the width of the clear uncalcified epiphyseal cartilage was determined with vernier calipers.

RESULTS

Solubilization of inclusion bodies with the cationic surfactant CTAC

The inclusion bodies containing rPGH were solubilized with CTAC and the soluble material was analysed by reversed-phase-h.p.l.c. and SDS/PAGE (Fig. 1). On the basis of h.p.l.c. profile area estimates, approx. 50% of the total protein was rPGH. The

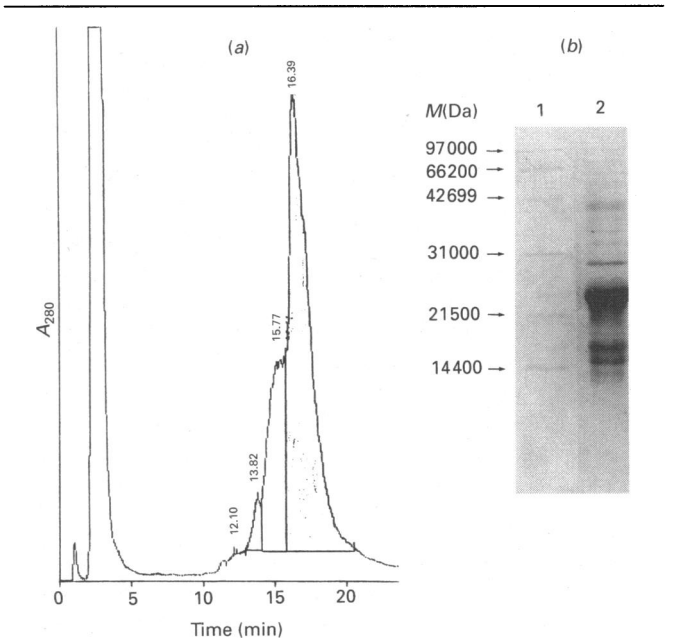

Fig. 1. Solubilization of rPGH from inclusion bodies

(*a*) Reversed-phase h.p.l.c. analysis (C_1 column) of rPGH solubilized from inclusion bodies with 5% (w/v) CTAC and 2% (v/v) 2-mercaptoethanol at 50 °C for 1 h. On the basis of relative peak area, rPGH (shaded) represents approx. 50% of the soluble protein fraction. The identification of the reduced rPGH peak was based on retention time, relative to a purified reduced rPGH standard as described previously [22]. (*b*) SDS/PAGE analysis (12.5% gel) of rPGH from inclusion bodies solubilized as in (*a*). Lane 1, standard molecular-mass (*M*) markers; lane 2, solubilized inclusion bodies. The reduced rPGH in lane 2 migrates with a molecular mass of approx. 24.5 kDa. For both (*a*) and (*b*), before analysis, solubilized protein was treated with a cation-exchange resin to remove CTAC.

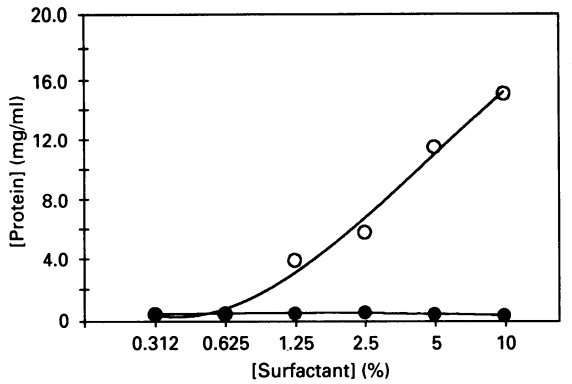

Fig. 2. Solubilization of inclusion bodies by CTAC (○) and CDA (●)

Solubilization of inclusion bodies by increasing concentrations of CTAC [$(CH_3)_3N^+C_{16}H_{33}$] and CDA [$(CH_3)_2NC_{16}H_{33}$]. Conditions of solubilization were as described in the Experimental section. Protein concentration was determined by h.p.l.c.

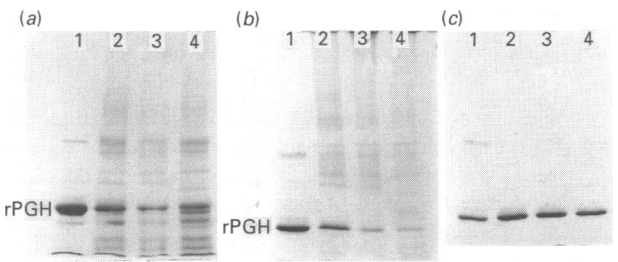

Fig. 3. SDS/PAGE analyses of urea-, GdmCl- and CTAC-solubilized and refolded rPGH

(*a*) SDS/PAGE analyses (12.5% gels) of reduced rPGH solubilized from inclusion bodies with urea, GdmCl or CTAC. Lane 1, reduced rPGH standard; lane 2, CTAC-solubilized rPGH; lane 3, GdmCl-solubilized rPGH; lane 4, urea-solubilized rPGH. (*b*) SDS/PAGE analyses of refolded rPGH preparations. Lane 1, oxidized rPGH standard; lane 2, CTAC-solubilized rPGH; lane 3, urea-solubilized rPGH; lane 4, GdmCl-solubilized rPGH. (*c*) SDS/PAGE analyses of refolded monomeric rPGH preparations after purification by DE-52 ion-exchange chromatography. Lane 1, oxidized rPGH standard; lane 2, CTAC-solubilized rPGH; lane 3, urea-solubilized rPGH; lane 4, GdmCl-solubilized rPGH. Further quantitative analyses of these gels is presented in Table 1.

historical basis behind selection of surfactant and conditions of solubilization will be elaborated in the Discussion. Briefly, solubilization was best achieved by using a 5% (w/v) or a 1:1 (w/w) ratio of CTAC to dry weight of inclusion body at pH 10.0 and at 50 °C for 30–60 min. These conditions resulted in maximal solubilization of inclusion body, estimated at 75–80% of dry weight. The inclusion bodies were normally solubilized to give 30–40 mg of protein/ml in solution. Solubilization conducted at elevated temperatures (40–50 °C) was found to decrease substantially the times for maximal recovery of rPGH from overnight (at 4 °C or 20 °C) to just 30 min at 50 °C.

After solubilization of the inclusion bodies, the positively charged CTAC (bound to protein or free in solution) was removed by using an anion-exchange resin at pH 10.0. Under these conditions, the rPGH carried a net negative charge and therefore did not adhere to the resin. During this CTAC 'stripping' procedure and particularly at high concentrations of protein (30–40 mg/ml), the use of 4–5 M-urea in the column

Table 1. Comparison of the use of CTAC, urea and GdmCl for the recovery of rPGH from inclusion bodies

	Assessment criteria	Recovery of rPGH: 7 M-urea	6 M-GdmCl	5% (w/v) CTAC
(*a*)	Total protein solubilized from inclusion bodies (mg/ml)*	19.5	20.0	15.5
(*b*)	Reduced rPGH (% of the total protein solubilized)†	20.0	38.0	43.0
(*c*)	Percentage of monomeric (i.e. 21.5 kDa) rPGH after refolding‡	12.5	20.0	30.0
(*d*)	Refolding efficiency (i.e. recovery of monomeric rPGH, %)§	62.5	52.0	70.0
(*e*)	Final recovery of monomeric rPGH after DE-52 purification (mg)‖	12.0	11.0	27.0

* Based on a Coomassie Blue (Bradford) total protein assay relative to a BSA standard.

† Based on laser-densitometer scan and integration of the appropriate reduced rPGH band areas from the SDS/polyacrylamide gel shown in Fig. 3(*a*).

‡ Based on laser-densitometer scan and integration of oxidized rPGH band area from SDS/polyacrylamide gel shown in Fig. 3(*b*).

§ Value is calculated as the percentage of oxidized monomeric rPGH divided by the percentage of reduced rPGH at solubilization [i.e. value in (c) divided by value in (b)].

‖ Based on approx. 200 mg (dry weight) of inclusion body as starting material.

buffer was useful in maintaining maximum solubility of rPGH during the critical transition phase between the 'surfactant-bound' and the 'surfactant-free' state. The use of urea therefore increased the recovery of soluble protein at this step, although it is not essential.

Solubilization of inclusion bodies by CTAC and CDA

The efficiency of solubilization of inclusion bodies by CTAC and a non-ionic form, CDA [$(CH_3)_2NC_{16}H_{33}$], was investigated in order to assess the relative importance of hydrophobic (16-carbon acyl side chain) and ionic interactions for solubilization. Under identical conditions, CDA was found to be ineffective for the solubilization of inclusion bodies for CDA concentrations up to 10% (w/v) (Fig. 2).

Comparison of CTAC, urea and GdmCl for solubilization and recovery of rPGH from inclusion bodies

Inclusion bodies containing rPGH were solubilized and the rPGH was refolded by using the CTAC, urea and GdmCl methods. SDS/PAGE analyses of the rPGH preparations after solubilization and refolding are shown in Figs. 3(*a*) and 3(*b*) respectively. The DE-52-purified monomeric rPGH preparations isolated from the refolded samples are shown in Fig. 3(*c*). In order to compare quantitatively the relative merits of the three approaches for solubilization and refolding of rPGH, the total concentration of protein solubilized and the refolding efficiency (i.e. recovery of monomeric rPGH at the expense of high-molecular-mass aggregates) were evaluated for each technique (Table 1). The final assessment criterion was net recovery of monomeric rPGH after DE-52 purification.

There were notable differences between the relative refolding efficiencies of the CTAC and the urea/GdmCl methods, CTAC being about 1.5 times more efficient for refolding rPGH than urea or GdmCl (Table 1). Overall, the CTAC method resulted in

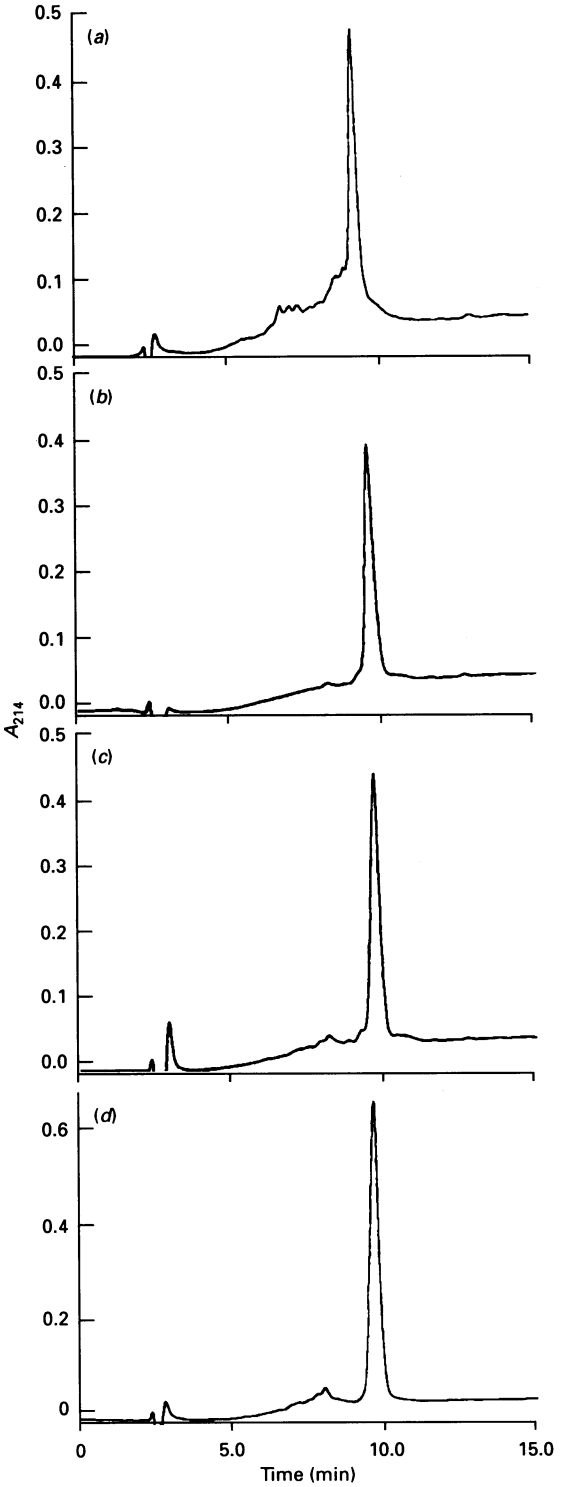

Fig. 4. Reversed-phase-h.p.l.c. analysis of rPGH preparations

Reversed-phase h.p.l.c. analysis of pituitary growth hormone (*a*) and DE-52-purified monomeric rPGH solubilized with (*b*) CTAC, (*c*) GdmCl and (*d*) urea. The rPGH preparations were solubilized, refolded and purified as described in the Experimental section. The average purity of the rPGH preparation was > 95%, as judged by h.p.l.c. and the four samples showed identical retention times.

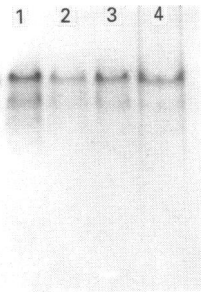

Fig. 5. Native PAGE analysis of purified rPGH preparations and pituitary growth hormone

DE-52-purified monomeric rPGH was analysed by PAGE in the absence of SDS. Lane 1, rPGH prepared by using CTAC; lane 2, rPGH prepared by using urea; lane 3, rPGH prepared by using GdmCl; lane 4, pituitary-derived growth hormone. A 12.5% acrylamide gel was used.

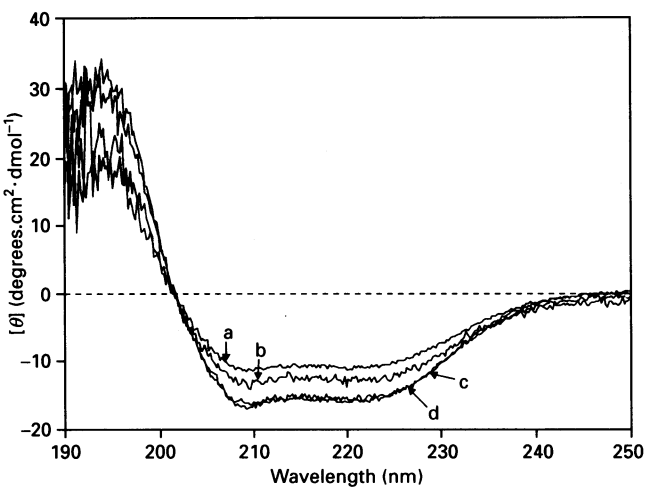

Fig. 6. C.d. analysis of purified rPGH preparations and pituitary growth hormone

C.d. spectra of purified pig pituitary growth hormone (a), and DE-52-purified, monomeric rPGH preparations solubilized with GdmCl (b), urea (c) and CTAC (d). The θ values are the calculated measures of the mean residue ellipticities in degrees·cm^2·mol^{-1}. Analysis was conducted using an AVIV 60DS instrument at a temperature of 25.8 °C, slit width of 0.07 with scanning from 250 nm to 195 nm in 0.2 nm shifts.

the highest refolding efficiency, although its efficiency of solubilization was approx. 20% less than urea or GdmCl. There were also differences in the percentage of reduced rPGH solubilized, with the CTAC and GdmCl methods respectively being most efficient at solubilizing rPGH. The overall yields of monomeric rPGH after DE-52 purification were greatest for CTAC- followed by urea- and GdmCl-solubilized material, being in the proportions of 2.25:1:0.9 respectively.

Characterization of rPGH preparations

The DE-52-purified monomeric rPGH preparations solubilized and refolded by the CTAC, urea and GdmCl methods were further characterized relative to each other or in comparison with purified pig pituitary growth hormone by reversed-phase-h.p.l.c., non-SDS/PAGE, c.d. spectroscopy, a competitive radioreceptor-binding assay and rat tibial bioassay.

Reversed-phase h.p.l.c. The CTAC-, urea- and GdmCl-derived rPGH preparations and pituitary growth hormone were essentially indistinguishable by h.p.l.c. with very similar retention times (Fig. 4) and with respective purities of 98%, 98%, 95% and 78%.

Non-SDS/PAGE. The relative heterogeneity of the rPGH and pituitary preparations was also assessed by native PAGE. Under

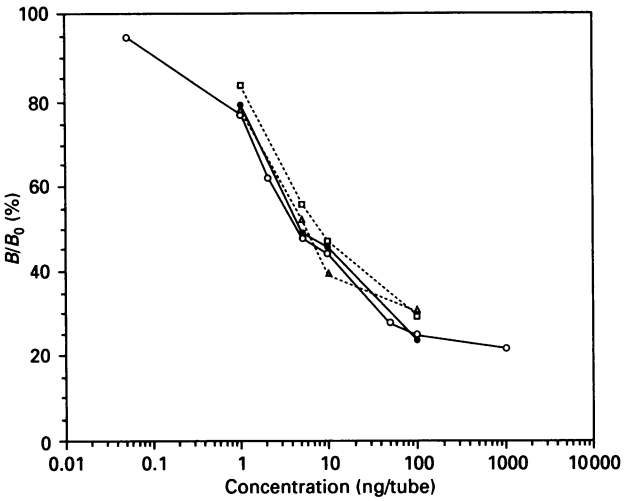

Fig. 7. Radioreceptor assay of rPGH preparations

Results of competitive binding of DE-52-purified monomeric rPGH preparations solubilized with urea (△), GdmCl (□) or CTAC (●) to the pig growth hormone receptor on liver membrane preparations, in the presence of an ^{125}I-labelled pituitary-derived pig growth hormone standard (○). Values shown are the means of two independent determinations plotted as percentage of counts bound/total (B/B_0) against ng of competing protein.

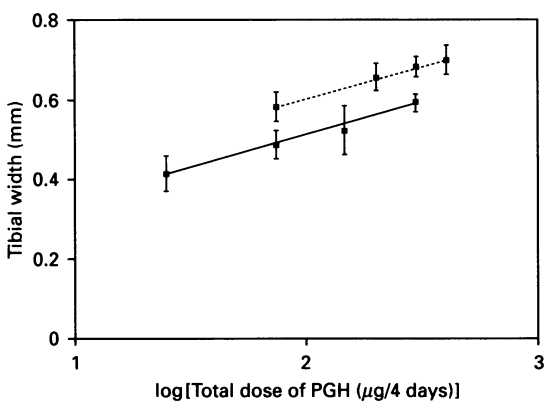

Fig. 8. Rat tibial bioassay of pig pituitary growth hormone and CTAC-solubilized rPGH

Results of hypophysectomized rat tibial bioassay of a DE-52-purified monomeric preparation of rPGH (■----■) and pituitary-derived pig growth hormone (■——■). The values shown are the means ($n = 8$) ± S.D. for each dosage. Linear regression (r^2) values of 0.98 and 0.99 and slopes of 1.62 and 1.61 for pituitary growth hormone and rPGH respectively were obtained. The control value for untreated rats was 4.10 ± 0.5 S.D.

these conditions, separation should reflect differences in molecular size and/or shape and/or charge, but most probably, the latter. In all cases, two major and occasionally a third very minor species of growth hormone were evident (Fig. 5). There were, however, some differences in the intensities of the different species depending on whether CTAC, GdmCl or urea preparations were examined. The ratios of the two major species on the basis of densitometric analyses were: 2.1:1 for the CTAC-derived rPGH, 2.5:1 for the urea-derived rPGH, 2.6:1 for the GdmCl-derived rPGH and 2.6:1 for the pituitary-derived growth hormone. These values would suggest no significant difference in heterogeneity between the different rPGH preparations.

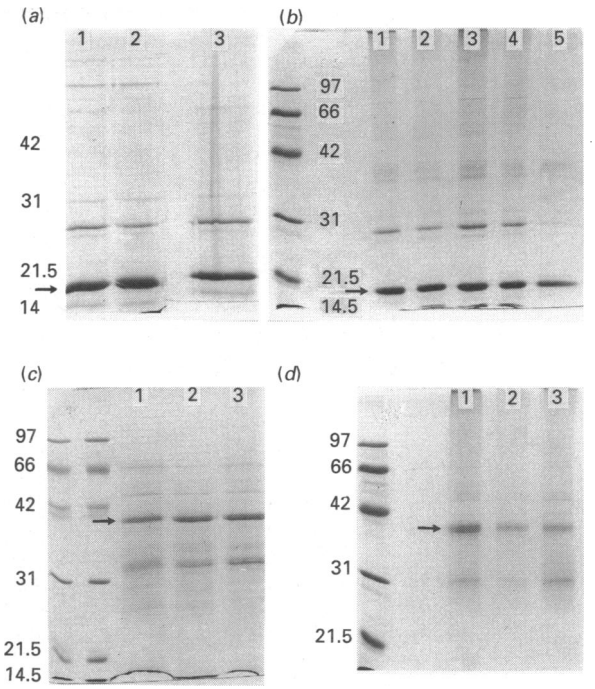

Fig. 9. SDS/PAGE analysis of urea-, GdmCl- and CTAC-solubilized and refolded rIL-1β and rIGF-II fusion protein

(*a*) SDS/12.5% polyacrylamide gel after electrophoresis of reduced rIL-1β (arrowed) solubilized from inclusion bodies. Lane 1, with urea; lane 2, with GdmCl; lane 3, with CTAC. Samples were run under reducing conditions. Standard molecular-mass markers (kDa) are shown. (*b*) SDS/12.5% polyacrylamide gel after electrophoresis of refolded rIL-1β preparations. Lanes 1 and 2, rIL-1β refolded at respectively 1.0 and 0.5 mg/ml after solubilization in urea; lanes 3 and 4, rIL-1β after solubilization in GdmCl; lane 5, rIL-1β refolded at 1.0 mg/ml after solubilization in CTAC. The refolded rIL-1β is arrowed. Standard molecular-mass markers (kDa) are shown. (*c*) SDS/PAGE analysis of rIGF-II fusion protein (arrowed) solubilized from inclusion bodies. Lane 1, with urea; lane 2, with GdmCl; lane 3, with CTAC. Samples were run under reducing conditions. Standard molecular-mass markers (kDa) are shown. (*d*) SDS/12.5% polyacrylamide gel after electrophoresis of refolded rIGF-II fusion protein preparations. Lane 1, rIGF-II refolded at approx. 1.0 mg/ml after solubilization in urea; lane 2, rIGF-II refolded after solubilization in CTAC; lane 3, rIGF-II refolded at 1.0 mg/ml after solubilization in GdmCl. The refolded rIGF-II is arrowed. Standard molecular-mass markers (kDa) are shown. Further quantitative analysis of the above gels is presented in Table 2.

C.d. analysis. The peptide band adsorption spectra (< 240 nm) of rPGH preparations and pituitary-derived growth hormone were examined by c.d. spectroscopy to compare secondary structure. The c.d. spectra of the three rPGH preparations in particular were very similar (Fig. 6), with the spectral curves for urea- and CTAC-derived rPGH being essentially indistinguishable. The relative proportions of α-helical content ranged from approx. 30 to 40%, with both the urea- and CTAC-derived samples containing 40% α-helix and the GdmCl- and pituitary-derived samples respectively 35% and 30% α-helix. The greater α-helical content was generally reflected in a more positive perpendicularly polarized band near 190 mm and a more negative $n-\pi^*$ band near 220 mm.

Radioreceptor assay. The similar (pig liver membrane) receptor-binding characteristics of CTAC, urea and GdmCl rPGH preparations were studied in a competitive assay relative to a commercially available ^{125}I-labelled pig pituitary-derived growth hormone (Fig. 7). The binding curves for CTAC-derived rPGH and the labelled pituitary growth hormone standard were very closely aligned, suggesting no significant differences in

binding affinity or alteration in specificity as the CTAC-derived rPGH displaced all the ^{125}I-labelled pituitary growth hormone. The other rPGH preparations showed similar behaviour, although there were minor variations with regard to the shapes of the urea- and GdmCl-derived rPGH displacement curves relative to pituitary growth hormone.

Rat tibial bioassay. The dose–response characteristics and therefore the relative potencies of pituitary-derived pig growth hormone and CTAC-derived rPGH were judged from the results of a hypophysectomized rat tibial bioassay (Fig. 8). The increase in width of the epiphyseal cartilage of the tibia has been demonstrated to be a sensitive and accurate test resulting in a straight-line log dose–response curve [15]. The use of CTAC-derived rPGH resulted in a straight-line dose–response curve parallel to, but laterally displaced relative to, that for the pituitary control (Fig. 8). For a test sample, with identical functional activity (biopotency), a straight-line curve coincident with the standard would be expected [15]. We observed a displacement of 0.55 log units corresponding to an approx. 3.5 fold increase in biopotency of rPGH over pituitary-derived growth hormone. (The pituitary-derived growth hormone used had an assigned potency of 1.5 i.u./ml based on an NIH-GB-B18 standard; M. R. Brandon, personal communication.) However, the single point of overlap between the two dose–response curves (first and last data points on the rPGH and pituitary-derived curves respectively) did not allow meaningful statistical analysis to establish confidence limits for potency.

Comparison of the use of CTAC, urea and GdmCl for recovery of rIL-1β and rIGF-II fusion protein from inclusion bodies

Two other recombinant proteins, sheep rIL-1β and an rIGF-II fusion protein expressed in *E. coli* as inclusion bodies were solubilized and refolded by using the CTAC, urea and GdmCl methods. SDS/PAGE analyses of reduced rIL-1β after solubilization and refolding are shown in Figs. 9(*a*) and 9(*b*) respectively. The rIL-1β, of molecular mass approx. 18 kDa, was clearly evident as the major protein species present. Similarly, SDS/PAGE analyses of solubilized and refolded rIGF-II fusion protein (arrowed), again the dominant species at solubilization and after refolding (molecular mass approx. 40 kDa), are shown in Figs. 9(*c*) and 9(*d*) respectively. All the results pertaining to 'rIGF-II' therefore relate to a fusion protein.

In order to compare quantitatively the relative merits of the three approaches to solubilization and refolding, the total concentrations of protein solubilized from inclusion bodies, the specific percentage of rIL-1β or rIGF-II solubilized and the recovery of monomeric protein at the expense of higher-molecular-mass aggregated forms [cf. Figs. 9(*b*) and (*d*)] after refolding, was evaluated by densitometric scanning of gels (results presented in Table 2). There were no significant consistent trends in the relative solubilization efficiencies or the percentage of specific protein solubilized from inclusion bodies by CTAC, urea or GdmCl. Differences with regard to relative refolding efficiencies (defined as percentage of oxidized monomeric, i.e. 18 kDa or 40 kDa protein species divided by the percentage of specific reduced protein solubilized) were more evident. CTAC-derived protein resulted in respectively 83% and 70% refolding effici-

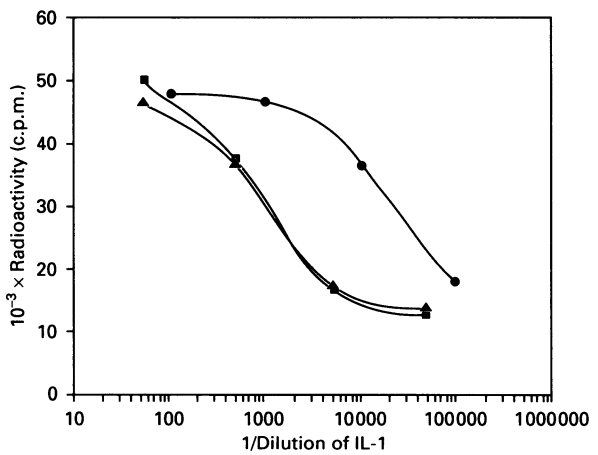

Fig. 10. Bioactivity assessment of rIL-1β preparations

Results of a proliferative cellular bioassay testing refolded IL-1β preparations initially solubilized in CTAC (●), GdmCl (■) or urea (▲). The *y*-values represent incorporation of [^{3}H]thymidine. The dilutions on the *x*-axis are plotted on a log scale. End-point titres of $1/10^3$ for both urea- and GdmCl- and $1/10^4$ for CTAC-solubilized and refolded rIL-β are based on the dilution corresponding to 50% maximal incorporation of [^{3}H]thymidine. The dilution values have been standardized to take into account the different starting concentrations of urea-, GdmCl- and CTAC-solubilized and refolded preparations. Background radioactivity was 8000 c.p.m.

Table 2. Comparison of the use of CTAC, urea and GdmCl for the recovery of (A) rIL-1β and (B) rIGF-II fusion protein from inclusion bodies

Assessment criteria		Recovery of rIL-1β or rIGF-II fusion protein: 7 M-urea	6 M-GdmCl	5% (w/v) CTAC
A				
(*a*)	Total protein solubilized from inclusion bodies (mg/ml)*	1.25	1.10	0.96
(*b*)	Reduced rIL-1β (% of the total solubilized protein)†	84.0	78.0	68.0
(*c*)	Refolding efficiency (i.e. recovery of monomeric rIL-1β, %) at respectively 0.2 mg/ml and 1.0 mg/ml of protein‡	70.0 50.0	74.0 60.0	83.0
B				
(*a*)	Total protein solubilized from inclusion bodies (mg/ml)*	1.45	1.37	1.25
(*b*)	Reduced rIGF-II (% of the total solubilized protein)§	30.0	46.0	40.0
(*c*)	Refolding efficiency (i.e. recovery of monomeric rIGF-II, %) after refolding at approx. 1.0 mg/ml‖	63.0	56.0	70.0

* Based on a Coomassie Blue (Bradford) total protein assay relative to a BSA standard.

† Based on laser-densitometer scan and integration of the appropriate reduced rIL-1β band areas from the SDS/polyacrylamide gel shown in Fig. 9(*a*).

‡ Value shown is based on laser-densitometer scan and integration of the appropriate oxidized rIL-1β band areas from SDS/polyacrylamide gel shown in Fig. 9(*b*) divided by the amount of starting reduced rIL-1β [i.e. values shown in (*b*)]. For the CTAC method rIL-1β was refolded only at a single concentration (1 mg/ml).

§ Based on a laser-densitometer scan and integration of the appropriate reduced rIGF-II band areas from the SDS/polyacrylamide gel shown in Fig. 9(*c*).

‖ Value shown is based on laser-densitometer scan and integration of the appropriate oxidized rIGF-II band areas from SDS/polyacrylamide gel shown in Fig. 9(*d*) divided by the amount of starting reduced rIGF-II [i.e. values shown in part (b)].

encies for rIL-1β and rIGF-II compared with values of respectively 50% and 60% and 56% and 63% for urea- and GdmCl-derived rIL-1β and rIGF-II respectively. Moreover, at least for rIL-1β, refolding efficiencies of CTAC-solubilized material were significantly superior at the higher protein refolding concentration of 1 mg/ml, suggesting that the CTAC-derived protein was much less susceptible to aggregation during refolding *in vitro*.

Bioactivity of rIL-1β preparations

The refolded CTAC-, urea- and GdmCl-derived rIL-1β preparations (cf. Fig. 9*b*) were assayed for bioactivity using an IL-1 dependent cell line [14]. After standardizing for rIL-1β content (on the basis of the refolding efficiencies shown in Table 2), the different rIL-1β preparations were titrated to end point. The urea- and GdmCl-derived rIL-1β preparations gave essentially identical [^{3}H]thymidine-uptake curves, clearly distinguishable from the CTAC-derived material (Fig. 10). End-point titres (defined as dilution at 50% of maximal binding, normally the point of inflection of the uptake curves shown in Fig. 10) of 1/1000, 1/1000 and 1/10000 were obtained for respectively urea-, GdmCl- and CTAC-derived rIL-1β, demonstrating the significantly greater potency (specific activity) of the CTAC-derived rIL-1β.

DISCUSSION

The cationic surfactant CTAC is a single-chain quaternary nitrogen compound, with a positively charged head group and a 16-carbon acyl side chain, allowing for predominantly hydrophobic interactions through its 'tail' and ionic interactions through its 'head'. On the basis of the significant differences in the capacities of CTAC and CDA (the dimethyl and therefore non-charged variant of the surfactant) to solubilize inclusion bodies, solubilization is likely to be mediated by some form of ionic interaction due to the positively charged head groups. The nature of the specific counter ion, for example Cl^- in the case of CTAC, does not appear to be critical as CTAB (with the Br^- counter ion) can be used interchangeably with CTAC. A model for the mode of action of CTAC would be that the detergent binds to partially unfolded proteins in inclusion bodies via predominantly hydrophobic interactions through its 16-carbon acyl side chain, with the subsequent solubilization of inclusion bodies resulting from the electrostatic repulsion between the positively charged head groups of the surfactant. This electrostatic repulsion under optimal conditions is apparently sufficient to overcome the associative forces within inclusion bodies. The validity of this model requires further investigation, but it is consistent with our results with CDA and with literature on the behaviour of CTAC during interaction with soluble proteins [16–18] where certain CTAC-bound proteins are thought to adopt rigid rod-like structures as a result of electrostatic charge repulsion.

The following observations were noted during development of the CTAC solubilization procedure used. (1) The efficiency of solubilization of rPGH appears to be essentially independent of pH and ionic strength. Inclusion bodies were successfully solubilized in both distilled water and 0.1 M-NaCl at pH 6.0–10.0 with comparable results. (2) The number of accessible binding sites on rPGH for CTAC is influenced by temperature (M. Cardamone, N. K. Puri, W. H. Sawyer, R. J. Capon & M. R. Brandon, unpublished work), thus the kinetics of inclusion bodies solubilization improve logarithmically from 20 °C to 50 °C. (3) The concentration of CTAC required for solubilization of inclusion bodies can, provided that it is greater than the critical micellar concentration, be varied over the range 0.5–5% (w/v). However, at low concentrations the time for maximal solubilization is impractical.

The development of a surfactant-based alternative to more common methods for solubilizing inclusion bodies was motivated by our need during large-scale production of rPGH for a simple low-cost relatively gentle and biocompatible alternative to urea and GdmCl [there were significant problems associated with handling and disposal (recycling) of hazardous and ecologically incompatible solubilizing agents]. CTAC was selected for evaluation as an example of a readily available quaternary ammonium compound. Its 16-carbon side chain and hence low critical micellar concentration of about 0.03% [19] relative to 14-carbon and 12-carbon derivatives was considered operationally advantageous, as relatively low concentrations (1–5%, w/v) were required for maximal solubilization of inclusion bodies. Furthermore, the cationic nature of CTAC (positively charged head group at pH 10.0) allowed its quantitative removal by a simple ion-exchange step under conditions where most proteins would be negatively charged and therefore not bind to a cation-exchange column. Successful and complete removal of CTAC was critical from the viewpoint of subsequent refolding of protein as well as for regulatory purposes. Finally, quaternary ammonium compounds were well known as being biocidal for bacteria thereby decreasing the stringency of processing conditions.

In order to establish the merits of the CTAC approach, a detailed comparison was undertaken with regard to existing approaches used to solubilize and recover recombinant proteins, as well as detailed structural and, where possible, bioactivity comparisons of the final product. These studies were conducted by using rPGH, rIL-1β and a rIGF-II fusion protein. In general, the efficiency of solubilization of inclusion bodies with 5% (w/v) CTAC was lower in comparison with 6 M-GdmCl and 7 M-urea. The consistent significant difference with the use of CTAC was the superior refolding efficiency, which for rPGH and rIL-1β ensured that the yield of monomeric material was usually higher than with urea and significantly higher than with GdmCl. In general, the behaviour and recoveries of CTAC-solubilized recombinant proteins were more closely aligned with results obtained with urea than with those obtained with GdmCl, the latter being operationally the inferior of the three approaches used.

Given the propensity of recombinant proteins solubilized from inclusion bodies to aggregate (particularly intradisulphide-bonded proteins such as rPGH and rIL-1β), low concentrations of urea and/or GdmCl (1–3 M) are almost invariably used to provide a weak denaturing environment in order to effect renaturation *in vitro* of proteins with adequate yield [2,4,6]. For CTAC-solubilized rPGH, rIL-1β and rIGF-II we obtained in all cases superior refolding efficiencies relative to refolding against 3 M-urea, while being able to renature against a simple aqueous buffer. This would strongly support the view that CTAC-solubilized proteins have a lower tendency for aggregation. A reason for this may be that 5% (w/v) CTAC, although effecting solubilization, causes less perturbation (denaturation) to protein secondary structure than 7 M-urea or 6 M-GdmCl, the latter being known to cause proteins to adopt a 'random coil' structure [20]. In contrast, there are reports in the literature that RNAase A, for instance, is not appreciably denatured by exposure to a closely related compound to CTAC, the 12-carbon chain surfactant, dodecyltrimethylammonium bromide [21]. It would be reasonable to expect that a protein that retained more of its native structure would subsequently be easier to renature to its original state. In support of this, our recent results [22] demonstrate that, in an environment specifically optimized to prevent aggregation due to aberrant disulphide bonding, up to 90% of the CTAC-solubilized rPGH can be obtained in a monomeric

and fully bioactive state by using a simple refolding solution free of denaturants.

In addition to process comparisons, we also characterized in detail the final DE-52-purified rPGH recovered by using CTAC, urea and GdmCl. On the basis of h.p.l.c. analyses, native PAGE, c.d. spectroscopy and radioreceptor-binding assay, the CTAC-solubilized product was closely comparable with, or superior to, urea- and GdmCl-based preparations. The CTAC-derived rPGH was bioactive and of greater potency and higher specific activity than the pig pituitary-derived growth hormone on the basis of a rat tibial assay. The latter is not surprising given the expected activity of a highly purified recombinant product as opposed to the heterogeneous natural pituitary-derived product (Fig. 4, relative purities of 98 % and 78 %). Although the relative biopotencies of the urea- and GdmCl-derived rPGH preparations were not tested, on the basis of our structural comparisons and receptor-binding data and the closely related secondary structure by c.d. spectroscopy, the CTAC-derived material should be at least equipotent with these.

The significantly enhanced biopotency of the CTAC-derived rIL-1β preparation, one $\log_{10}$ dilution over urea- and GdmCl-derived material, was also noteworthy. The reasons for this are not clear in the absence of detailed structural characterization of the final products.

We conclude from the results presented here that CTAC represents a viable new alternative for recovering recombinant proteins from inclusion bodies such as rPGH and rIL-1β, with no major structural alterations and at least equal or usually enhanced yield and/or biopotency relative to the use of urea or GdmCl. In addition, the use of CTAC offers, in our view, several significant practical advantages during processing, particularly with regard to industrial-scale production of recombinant proteins.

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REFERENCES

1. Sharma, S. K. (1986) Separ. Sci. and Technol. **21(8)**, 701–726
2. Marston, F. O. (1986) Biochem. J. **240**, 1–12
3. Kane, J. F. & Hartley, D. L. (1988) TIBTECH **6**; 95–101
4. Schein, C. H. (1989) Biotechnology **7**, 1141–1149
5. Mitvaki, A. & King, J. (1989) Biotechnology **7**, 690–697
6. Schein, C. H. (1990) Biotechnology **8**, 308–317
7. Andrews, A. E., Barcham, G. J., Brandon, M. R. & Nash, A. D. (1991) Immunology **74**, 453–460
8. Yarrantoh, G. T., Wright, E., Robinson, M. K. & Humphreys, G. O. (1984) Gene **28**, 293–300
9. O'Mahoney, J. V. & Adams, T. E. (1989) Nucleic Acids Res. **17(13)**, 5391–5392
10. Smith, D. B. & Johnsen, K. (1988) Gene **67**, 31–40
11. Yang, J. T., Wu, C-S. C. & Martinez, H. M. (1986) Methods Enzymol. **140**, 228–233
12. Chan, J. S. D., Robertson, H. A. & Friesen, H. G. (1978) Endocrinology **102**, 632–640
13. Baxter, R. C., Zaltsman, Z. & Turtle, J. R. (1982) Endocrinology **114**, 1893–1901
14. Gearing, A. J. H., Bird, C. R., Bristow, A., Poole, S. & Thorpe, R. (1987) J. Immunol. Methods **99**, 7–12
15. Li, C. H. (1977) in Hormonal Proteins and Peptides: Growth Hormones and Related Proteins (Li, C. H., ed.), vol. 4, pp. 2–41, Academic Press, London
16. Nozaki, Y., Reynolds, J. A. & Tanford, C. (1974) J. Biol. Chem. **249(14)**, 4452–4459
17. Osterman, L. A. (1984) in Methods of Protein and Nucleic Acid Research, chapter 1, p. 70, Springer-Verlag, Berlin, Heidelberg, New York and Tokyo
18. Subramanian, M., Sheshadri, D. S. & Venkatatta, M. P. (1984) J. Biochem. (Tokyo) **95**, 413–421
19. Akin, D. T., Shapira, R. & Kinkade, J. M. (1985) Anal. Biochem. **145**, 170–176
20. Pace, C. N. (1986) Methods Enzymol. **131**, 266–280
21. Jones, M. N., Skinner, H. A., Tipping, B. & Wilkinson, A. (1973) Biochem. J. **135**, 231–236
22. Puri, N. K. (1991) FEBS Lett. **292**, 187–190